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Published in:
Mechanisms of Ageing and Development

DOI:
10.1016/j.mad.2005.08.008

Publication date:
2006

Available online at www.sciencedirect.com
SCIENCE DIRECT
Mechanisms of Ageing and Development 127 (2006) 85–87
mechanisms of ageing and development
www.elsevier.com/locate/mechagedev

Short communication

Continuous growth of telomerase-immortalised fibroblasts: How long do cells remain normal?

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Received 25 July 2005; accepted 24 August 2005
Available online 5 October 2005

Abstract

Previously, we reported successful immortalisation following hTERT introduction in primary human fibroblasts, strain VH25. Since one subclone in that study developed some abnormalities, we decided to study eight additional independent immortalised clones to get an indication of the frequency and type of abnormalities that develop after hTERT-mediated immortalisation. We show that although some cell lines can maintain a normal phenotype for 500 population doublings (PDs), in four clones after 150–300 PDs changes developed in basal and radiation-induced p53 and $p21^{WAF\text{-}1,CIP\text{-}1}$ levels. Our experiments demonstrate that, after prolonged culture, cells with abnormalities in cell cycle control parameters can take over the population. This calls for caution when working with hTERT-immortalised cells in vitro as well as in vivo.

Keywords: Telomerase; Immortalisation; hTERT; p53; Human fibroblast

After the initial demonstration that the introduction of human telomerase (hTERT) in normal human cells is sufficient to immortalise them (Bodnar et al., 1998), a number of reports have been published on the effects of immortalising cells with hTERT. No changes in phenotype were reported, and hTERT immortalised cells appeared to provide an infinite supply of normal human cells (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999; Nakamura et al., 2002). We recently reported our results with hTERT immortalised human foreskin fibroblasts (VH25) in response to ionising radiation (Kampinga et al., 2004) and showed that these cells retained their normal phenotype for the first few 100 population doublings (PDs) while VH25 fibroblasts senesce at PD 40–45. However, we observed one subclone at PD 310 which showed tetraploidy and altered response in radiation sensitivity (Kampinga et al., 2004). To further investigate the frequency of occurrence of an abnormal phenotype, we continued to grow seven additional independent hTERT immortalised clones during 80–150 weeks, corresponding to 250–550 PDs. At several time points growth characteristics and cell cycle parameters were analysed (see Table 1). As we reported earlier, during the first 100 PDs no alterations compared to the primary cells (VH25) were seen in terms of radiosensitivity (clonogenic assay), DNA double-strand break repair (as measured by PFGE), radiation-induced increases in p53 and p21 expression, and the G1/S and G2/M cell cycle checkpoints. However, after 150–300 PDs four out of eight independent immortal clones did show changes in some of these parameters. Three cell lines showed changes in p53 and $p21^{WAF\text{-}1,CIP\text{-}1}$ expression (Fig. 1; Group B) with or without changes in ploidy (Table 1; Group B). One other line became tetraploid without obvious changes in p53 and $p21^{WAF\text{-}1,CIP\text{-}1}$ expression (Table 1; Group C). The other four clones (Group A) still retained the characteristics of the wild type cells after 300–500 PDs, except for growth rates which increased for all eight clones from 2 to 3 PDs/week to about 4 PDs/week (Fig. 2). Although there is no significant difference in growth rate seen between the normal clones and the clones which developed abnormalities in cell cycle control (Group B) or ploidy (Group C) we observed an increase in growth rate before the onset of detectable abnormalities. We previously determined (Kampinga et al., 2004) that the mitotic index for one strain (K20) increased from about 85% in growing VH25 cells to about 95% in K20 cells at PD 300, which may be one explanation for the

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0047-6374/$ – see front matter
doi:10.1016/j.mad.2005.08.008

Table 1
Properties of eight h-TERT immortalised fibroblasts clones

Group	Cell	PD	PD/week	Ploidy	$p16^{INK4a}$	Irradiation response after 6 Gy		
						p53	$p21^{WAF-1,CIP-1}$	Survival (%)
WT	VH25	45	2.5	2*n*	wt	wt	wt	0.8
A	K21	470	3.6	2*n*	up/wt	wt	wt	2.0
	K22	380	3.8	2*n*	ND	wt	wt	0.3
	K25	320	3.1	2*n*	ND	wt	wt	1.0
	K26	320	3.6	2*n*	up/wt	wt(+)	wt	1.0
B	K15	320	3.4	2*n*	up/wt	+	–	10.3
	K20	330	5.2	4*n*	ND	+	–	20.0
	K23	260	3.8	2*n*–4*n*	wt	+	–	12.4
C	K19	290	2.6	4*n*	ND	wt	wt	0.1

The cells were immortalised by transfection at PD 23 with pGRN145 (Geron Corp., Menlo Park, USA). The parent line VH25 has a maximal number of 45 PDs before it senesces. In the immortalised clones, PD indicates the population doubling number reached at the end of our experiment. Growth rate (PD/week), ploidy and cell cycle checkpoint controls are shown at the indicated PD. Four clones (Group A) still retained their wild type characteristics while three clones (Group B) developed changes in (radiation induced) cell cycle control with or without tetraploidy. Tetraploidy without cell cycle control changes was detected in only one clone (Group C). Ploidy was tested by FACS analysis and survival after 6 Gy of γ-rays was assayed using the clonogenic assay (Kampinga et al., 2004).

observed increase in growth rate of the cultures. The increasing growth rate during prolonged culture did not result in loss of contact inhibition in any of the eight clones as determined by the soft agar assay (data not shown).

Our analysis reveals that three cell lines show an increase in constitutive p53 expression paralleled by a loss in constitutive and radiation-inducible $p21^{WAF-1,CIP-1}$ expression (Fig. 1; Group B). It may be possible that due to the absence of $p21^{WAF-1,CIP-1}$ these cells allow elevated expression of p53 without inducing cell cycle arrest. This may also explain the reduced radiosensitivity as was demonstrated with the clonogenic cell survival assay. Alternatively, p53 overexpression may be caused by mutations in the p53 gene locus, rendering the genes inactive, as occurs in many cancers (Soussi and Lozano, 2005). The presence of tetraploidy by itself seems to have no effect on radiosensitivity as was demonstrated in K19 (Table 1).

The increase in p53 expression may be due to stress caused by DNA damage during prolonged culture and in normal, $p21^{WAF-1,CIP-1}$-expressing populations these cells would have been eliminated by growth-arrest (Ben Porath and Weinberg, 2005; Campisi, 2005). Due to the inability to express $p21^{WAF-1,CIP-1}$ these cells can now enter mitosis with unrepaired DNA giving rise to genomic instability which in turn may lead to a transformed phenotype. Such cells will have a growth advantage and will take over the population.

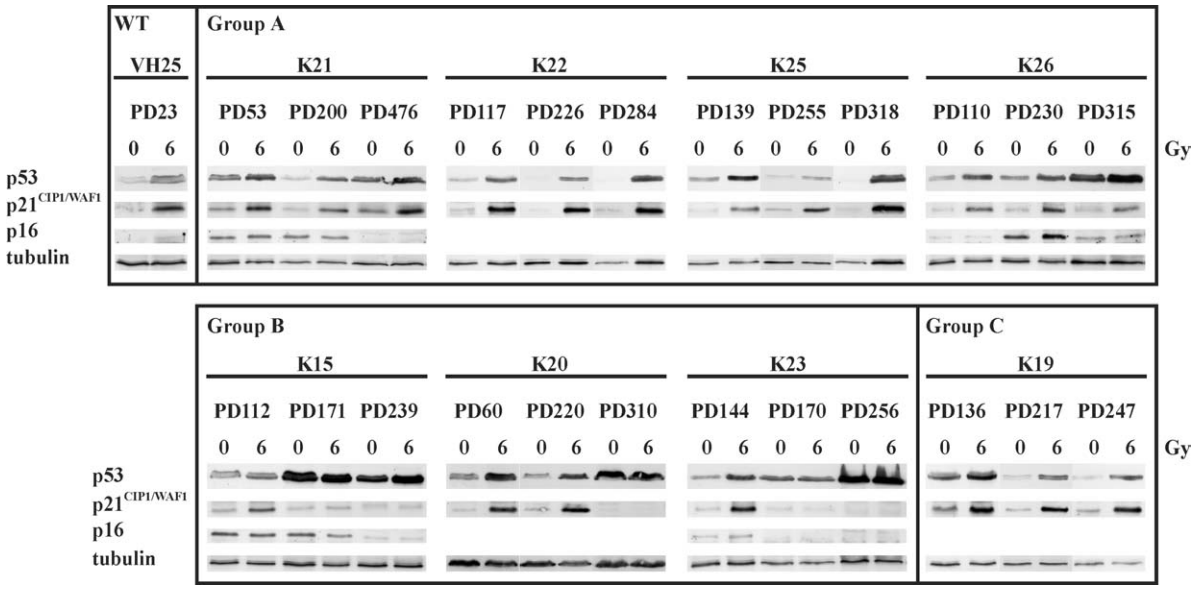

Fig. 1. Western blotting of p53, $p21^{WAF-1,CIP-1}$ and $p16^{INK4a}$ in hTERT-immortalised fibroblasts, non-irradiated (0 Gy) and 6 h after γ-irradiation (6 Gy). Shown are non-transfected cells (VH25) and the three groups A–C of the immortalised fibroblasts at the indicated PD-number. Group A represents the clones which still retained the wild type characteristics. In Group B, the cells developed abnormalities in cell cycle control parameters, either with or without tetraploidy. The clone in Group C developed tetraploidy only. The method used is as described in Kampinga et al. (2004) using the p53 (DO-1) and p16 (C-20) antibodies of Santa Cruz Biotechnology, Santa Cruz, USA and $p21^{WAF-1}$ antibody (AB-1) of Oncogene, Amsterdam, The Netherlands. Detection of γ-tubulin (by γ-tubulin antibody T6557, Sigma, St. Louis, MO, USA) was used to monitor loading variation.

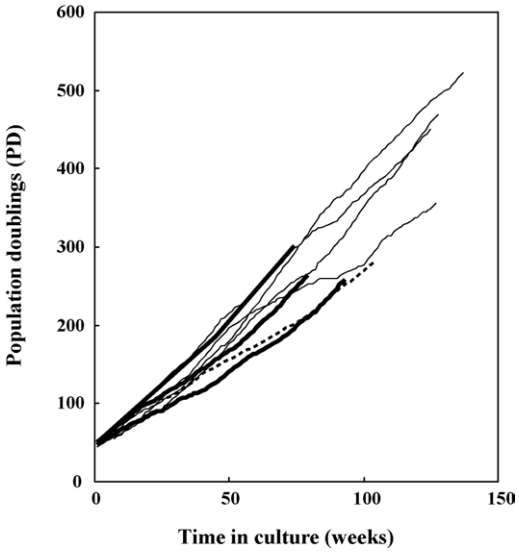

Fig. 2. Growth curves of hTERT immortalised fibroblasts: Group A (thin lines) still shows wild type characteristics; Group B (thick lines) started showing abnormalities in cell cycle control after 150–300 PDs with or without developing tetraploidy; Group C (dashed line) is tetraploid only. The wild type cells (VH25) stopped growing at 45 PD.

The $p16^{INK4a}$ expression level in the primary foreskin fibroblasts (VH25) is low. In some of the immortalised clones, it increased initially after the hTERT insertion, but after prolonged culture it decreased to levels comparable to the wild type (Fig. 1). No correlation of $p16^{INK4a}$ levels was observed with the development of abnormalities in the other cell cycle parameters or ploidy. It is of note that in all clones $p16^{INK4a}$ is reduced at the later PDs. This may imply a reduction in stress induced cell cycle arrest.

Changes in cell cycle parameters after immortalisation with hTERT were reported by others as well. Noble et al. (2004) observed a mutation of the p53 gene in one out of three immortal clones. Expression of $p16^{INK4a}$ was found to be elevated after immortalisation but gradually disappeared during the accelerated growth phase (Milyavsky et al., 2003; Noble et al., 2004). Our data for eight independent clones extend the observation that after prolonged growth, a culture is being overtaken by cells which have a growth advantage due to altered p53 or $p16^{INK4a}$ responses. These altered responses prevent the cells from entering cell cycle arrest when they experience stress, such as nutrient deprivation or oxidative damage (Ben Porath and Weinberg, 2005; Campisi, 2005). Selection for these altered responses is a stochastic process, which will partly depend on the level of experienced stress during culturing, and this may explain why in some cultures these changes occur shortly after immortalisation and why other cultures appear normal even after several hundreds of PDs.

It is now clear that, while initially it was believed that hTERT immortalised cells maintain a normal phenotype (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999; Nakamura et al., 2002), prolonged culturing will lead to cells with abnormal behaviour (Milyavsky et al., 2003; Noble et al., 2004; this work). Although immortalising fibroblasts by reconstitution of active telomerase can be a reliable manner to generate a large number of cells similar to primary cells, monitoring these cells remains important, since during continuous growth cells with altered characteristics can overtake the culture. For this reason, the use of hTERT for the immortalisation of cells for in vivo use needs to be considered with great caution.

Acknowledgements

This work was supported by a Grant 7.9.2 from the Interuniversitairy Institute for Radiopathology and Radiation Protection (IRS) awarded to H.H.K. The authors wish to thank the laboratory technicians who were involved in the cell culture of the clones and Henk Moes for help with the flow cytometric analysis.

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